Contributions of MS metabolomics to gilthead sea bream (*Sparus aurata*) nutrition. Serum fingerprinting of fish fed low fish meal and fish oil diets

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ABSTRACT

The aim of this study was to evaluate the impact of fish meal (FM) and fish oil (FO) replacement by plant proteins and oils in the serum metabolome of two-year old gilthead sea bream (*Sparus aurata*) fed from early life stages with control and experimental diets. Randomly selected fish were overnight sampled and clotted serum was used for metabolomics fingerprinting by means of ultra-high performance liquid chromatography coupled to quadrupole time-of-flight mass spectrometry. More than 12,500 different *m/z* ions were detected, and Partial Least Squares-Discriminant analysis separated fish fed control and plant-based diets, with a 71% of variance explained and 44% of variance predicted by the two first components. After variable importance in projection (VIP) and Benjamini-Hochberg test correction filtering, 50 endogenous compounds were elucidated as highly discriminant features of dietary treatment. Most of them were lipid-related compounds and reflected the different fatty acid composition of dietary oils, whereas changes in N-acyl taurines, cytidine and nucleoside related compounds would indicate changes in tissue repair and DNA degradation processes. Untargeted analysis also identified some exogenous compounds as markers of marine and vegetable raw materials. In the case of hercynine (antioxidant fungi and mycobacteria product), this was exemplified by a close lineal association between circulating and feed levels. Targeted approaches were focused on vitamins and a clear reduction of B_{12}, indirectly assessed via methylmalonic acid levels, was found in fish fed vegetable diets. Conversely, serum riboflavin (B_2) and pantothenic acid (B_5) levels were consistently increased, which highlighted the close link between nutrition and gut microbiota.

Keywords: Fish nutrition; liquid chromatography; mass spectrometry; metabolomics; vitamins; microbiota; plant-based diets.

1. Introduction

Current stagnation of fish meal (FM) and fish oil (FO) production from wild fisheries limits further growth of aquaculture (Tacon and Metian, 2015). The most obvious alternatives are the plant ingredients, which are a common practice in salmonids and marine fish to reduce the reliance of European aquaculture on marine fishery resources. Major progress in this way has been achieved within AQUAMAX, and ARRAINA EU projects and data on key performance indicators clearly indicate that alternative feeds with less than 7% marine ingredients support the maximum growth of gilthead sea bream (*Sparus aurata*) from early life stages to completion of sexual maturation (Benedito-Palos et al., 2016; Simó-Mirabet et al., 2018). It is also noteworthy that plant-based diets did not have a negative impact on the shelf life of gilthead bream, trout or carp as high quality foods (Grigorakis et al., 2018). Also, both in salmon and gilthead sea bream, no transfer from feeds to edible fillets was found for regulated mycotoxins, pesticides and persistent organic pollutants with the current plant-based diet formulations (Berntssen et al., 2005, 2010; Nácher-Mestre et al., 2009; 2015; Bell et al., 2012; Portolés et al., 2017). However, regardless of fish fatty acid (FA) biosynthetic capabilities, the use of plant-based diets is associated with a reduced content of n-3 long-chain poly-unsaturated FAs (PUFA) in the meat of farmed fish (Benedito-Palos et al., 2009; Liland et al., 2013, Ballester-Lozano et al., 2016; Turchini et al., 2018).

Other drawback effects of plant-based diets in marine farmed fish are related to changes in fish health and stress resilience (Montero and Izquierdo, 2010). Certainly, the magnitude and persistence of high plasma cortisol levels after crowding exposure is increased in juveniles of gilthead sea bream fed vegetable oils (Ganga et al., 2011), although a lower risk of oxidative stress in these challenged fish is also inferred (Pérez-Sánchez et al., 2013b). However, below the threshold level for the theoretical requirements in essential FAs, high inclusion levels of vegetable oils allow a faster disease progression in juveniles of gilthead sea bream challenged with the intestinal parasite *Enteromyxum leei* (Estensoro et al., 2011; Calduch-Giner et al., 2012). A possible cause are the nutritionally-mediated changes on the intestinal profile of mucins, mucosal immunoglobulins (*IgT*) and other immune-relevant genes of either diagnostic or predictive value (Calduch-Giner et al., 2012; Pérez-Sánchez et al., 2013c; Piazzon et al., 2016), which revealed a pro-inflammatory condition affecting also the integrity of

the intestinal barrier (Estensoro et al., 2016) and the composition of gut microbiota and intestinal mucus proteome (Piazzon et al., 2017). From these studies, however, it was also conclusive that most of these disturbing effects are reversed by the supplementation of plant-based diets with sodium butyrate, resulting in improved diseases outcomes in fish challenged with *E. lee*i and the bacteria *Photobacterium damselae subsp. piscicida* (Piazzon et al., 2017). Experimental evidence also indicates that diets enriched with medium-chain fatty acid salts (sodium heptanoate, sodium dodecanoate) have a positive impact on feed intake and energy metabolism of juvenile fish reared under sub-optimal conditions (Simó-Mirabet et al., 2017; Martos-Sitcha et al., 2018), although possible mechanisms still await full elucidation.

Very often, the application of targeted analyses is the prevailing strategy for qualitative and quantitative detection of different biomarkers. However, this strategy restricts the possibilities to detect other unpredictable effects that could result directly or indirectly from the changes in diet composition. This limitation has encouraged the development and application of new and powerful analytical approaches to face the complexity of this problem and to improve the chance to detect unanticipated effects. Currently a promising new "omic" approach is metabolomics, which aims to use profiles of low-molecular weight metabolic entities (usually < 1,000 Da) to identify biomarkers indicative of specific conditions and particular metabolic pathways. The novelty of this approach in aquaculture research is highlighted in the review article of Alfaro and Young (2018). In particular, nuclear magnetic resonance (NMR)-based lipid fingerprinting allows to precisely classify wild and farmed gilthead sea bream based on their muscle lipid composition (Melis et al., 2014). In another gilthead sea bream study, Robles et al. (2013) measured over 80 metabolites from fish intestine samples using a high-performance liquid chromatography-mass spectroscopy (HPLC–MS) platform. Although both analytical platforms rely on wide-untargeted approaches, MS allows retrospective analysis and a higher sensitivity and resolution power (Castro-Puyana and Herrero, 2013). Indeed, we have detected more than 15,000 *m/z* ions in the serum of gilthead sea bream by means of ultra-high performance liquid chromatography (UHPLC) and high resolution MS (HRMS) (Gil-Solsona et al., 2017). The same platform has been used in the present study to analyse fish from the eight-months feeding trial of Benedito-Palos et al. (2016). That study was prolonged, and herein data on wide- and targeted-serum metabolome were used to underline the effects of alternative feeds in two-year old fish fed experimental diets from early life stages.

2. Materials and methods

2.1. Reagents and chemicals

HPLC-grade methanol (MeOH), HPLC-supergradient acetonitrile (ACN), sodium hydroxide (> 99%), ammonium hydroxide (NH_4OH) and ammonium acetate (NH_4Ac) were obtained from Scharlab (Barcelona, Spain). HPLC-grade water was obtained from a Milli-Q water purification system (Millipore Ltd., Bedford, MA, USA). Leucine-enkephalin (mass-axis calibration), formic acid (mobile phase modifier), N,N-dimethyl L-histidine (reagent grade), methyl iodine (reagent grade) and tetrabuthylammonium acetate (reagent grade) were purchased from Sigma-Aldrich (Saint Louis, MO, USA).

2.2. Diets

Four experimental diets were formulated and produced by BioMar (Brande, Denmark). All diets were isonitrogenous, isolipidic and isoenergetic and met all known nutritional requirements of gilthead sea bream. FM was included at 23% in the D1 (control) diet and at 3% in the other three experimental diets (D2, D3 and D4). Fish hydrolysate (CPSP) was added at 2% in all diets. Added oil was either FO (D1 diet) or a blend of vegetable oils (1:1 ratio of rapeseed oil: palm oil), replacing 58% (D2 diet) and 84% (D3 and D4 diets) FO. A commercial butyrate preparation (BP-70®, NOREL) was added to the D4 diet at 0.4%. All diets contained histidine (0.14%), antioxidants (0.045%) and a mineral-vitamin mix (0.5%). Lysine, methionine, choline, lecithin and monocalcium phosphate were balanced in D2, D3 and D4 diets to the values of the control diet **(**Table 1).

2.3. Animal care and sampling

Juvenile fish (15 g initial average body weight) of Atlantic origin (Ferme Marine de Douhet, Ile d'Oléron, France) were fed control and experimental diets in the indoor experimental facilities of the Institute of Aquaculture Torre de la Sal (IATS-CSIC, Spain). Fish were allocated in 2,500 L tanks in triplicated groups (150 fish/tank), and each one was fed one of the experimental diets for 16 months from May 2013 to August

2014. The number of fish per tank was progressively reduced by periodical samplings, maintaining the rearing density below 15 kg/m^3. Oxygen content of outlet water remained higher than 75% saturation and day-length and water temperature followed natural changes at IATS-CSIC latitude (40° 5'N; 0° 10'E). At time of sampling, actively fed fish (3-4 fish per tank to achieve 10 fish per diet) were sampled following overnight fasting for blood and tissue collection. Liver and visceral adipose tissue were extracted and weighed. Blood was taken from caudal vessels with vacutainer tubes with a clot activator, allowed to clot for 30 min at room temperature, and then centrifuged at 1,300 *g* for 10 min. The obtained samples were stored at -20ºC until analysis.

All procedures were approved by the IATS Ethics and Animal Welfare Committee according to national (Royal Decree RD53/2013) and EU legislation (2010/63/EU) on the handling of animals for experiments.

2.4. UHPLC-HRMS

The analytical procedure was similar to that described elsewhere by Gil-Solsona et al. (2017). Briefly, serum samples were deproteinized with ACN and one supernatant aliquot was used for hydrophilic interaction liquid chromatography (HILIC). Another aliquot was evaporated to dryness and re-dissolved in MeOH 10% for reversed phase (RP) analysis. Quality control (QC) samples were prepared by pooling 50 µL of each sample extract. Extracts (10 µL) were injected in HILIC and RP in both positive and negative ionization modes (0.7 kV and 1.5 kV capillary voltages, respectively) in a hybrid quadrupole time-of-flight mass spectrometer (Xevo G2 QTOF, Waters, Manchester, UK) with a cone voltage of 25 V, using nitrogen as both desolvation and nebulizing gas.

The HILIC separation was performed using a mix of ACN:H_2O (95:5, v/v) as weak mobile phase (A) and H_2O as strong mobile phase (B) both in 0.01% formic acid (HCOOH) and 10 mM NH_4Ac. The percentage of B was changed as follows: 0 min, 2%; 1.5 min, 2%; 2.5 min, 15%; 6 min, 50%; 7.5 min, 75%; and finally at 7.51 min, 2%, with a total run time of 10 min, for both ESI+ and ESI-. For RP separation, the weak mobile phase (A) was H_2O with 0.01% HCOOH and the strong mobile phase (B) was MeOH with 0.01% HCOOH. The B percentage was changed from 10% at 0 min, to 90% at 14 min, 90% at 16 min and 10% at 16.01 min, with a total run time of 18 min for both ESI+ and ESI-. In order to obtain a better resolution among isomers of free FAs

and phospholipids, aliquots of RP samples were fortified at 50 mM with tetrabuthylammonium acetate (TBA) and injected with the following gradient: A: H_2O 0.01% HCOOH, B: MeOH 0.01% HCOOH; The percentage of B was maintained at 70% during the first 5 min and changed from 70% at 5 min, to 80% at 8 min, 85% at 12 min, 95% at 15 min, 100% at 22 min and 70% again at 22.01 min with a total run time of 24 min for both ESI+ and ESI-.

2.5. Untargeted Data Processing

LC-MS data were processed using XCMS R package (https://xcmsonline.scripps.edu/) with *Centwave* algorithm for peak picking (peak width from 5 to 20 s, S/N ratio higher than 10 and mass tolerance of 15 ppm), followed by retention time alignment, peak area normalization (mean centering), log 2 applying (to avoid heteroscedasticity) and Pareto scaling. For elucidation purposes, fragmentation spectra of features of interest were compared with reference spectra databases (METLIN, http://metlin.scripps.edu; Human Metabolome DataBase, http://www.hmbd.ca; MassBank, http://www.massbank.eu). For unassigned metabolites, *in silico* fragmentation software (MetFrag, http://msbi.ipb-halle.de/MetFrag), with subsequent searches through Chemspider (http://www.chemspider.com) and PubChem (https://pubchem.ncbi.nlm.nih.gov) chemical databases, was employed.

2.6. Targeted analysis

The retrospective analysis of data acquired in MS^E mode served for the refined search of additional relevant compounds. This procedure consisted in the search of the *m/z* ratio (parent ions) of the metabolites of interest in the LE function, as well as product ions obtained from MS/MS spectrum online databases (METLIN and Human Metabolome DataBase) in the HE function. In the case of vitamins and related-compounds, fat-soluble vitamins were not directly analysable in serum, and their related metabolites were analysed as retinol phosphate for vitamin A, 25-hydroxyvitamin D_3 for vitamin D_3, α-Carboxyethylhydroxychroman for vitamin E and menaquinone for vitamin K_2 (Tai et al., 2010; Lebold et al., 2012; Karl et al., 2014). Water-soluble

vitamins were directly analysed (B_1, B_2, B_5, B_6, B_7 and C) with the exception of B_{12}, which was indirectly assayed as methylmalonic acid (MMA) (Lewerin et al., 2003).

Targeted analysis was also applied for hercynine, a betaine compound synthetized by fungi and mycobacteria. This exogenous compound was analysed in feeds and serum samples, using a hercynine standard synthetized as described elsewhere (Khonde and Jardine, 2015). In the case of feed samples, the analytical protocol included a polar extraction procedure previously employed in our laboratory for animal by-products (Nácher-Mestre et al., 2016). Briefly, 2.5 g of feeds were extracted with 10 mL H_2O:ACN (20:80) 0.1% HCOOH, centrifuged and supernatant (5 mL) was passed through an OASIS WCX SPE cartridge previously cleaned with 6 mL MeOH and 6 mL of Milli-Q H_2O. Sample was loaded, cleaned with 6 mL of MeOH:H_2O (1:1) and finally eluted in 3 mL of 2% formic acid in methanol. The feeds samples were then lead to dryness and diluted in 200 µL of Milli-Q H_2O to continue with MS analysis.

2.7. Statistical analysis

Data on growth performance and targeted analysis were analysed by one-way ANOVA followed by the Student Newman–Keuls test ($P < 0.05$). After data preprocessing of untargeted metabolomics, multivariate analysis was performed to find discriminative features among groups by means of the EZ-Info software (Umetrics, Sweden). First, Principal Component Analysis (PCA) was used to ensure the absence of outliers and the correct classification of QCs after normalization. Then, all the four experimental groups were joined in a single file and Partial Least Squares-Discriminant Analysis (PLS-DA) was conducted to maximize the separation of dietary groups. The contribution of *m/z* features to the PLS-DA model was assessed by means of variable importance in projection (VIP) measurements. A VIP score > 1 was considered an adequate threshold to determine discriminant variables in the PLS-DA model (Wold et al., 2001; Li et al., 2012; Kieffer et al., 2016). Additionally, orthogonal PLS-DA (Wiklund et al., 2008) with a high threshold (P [corr] > 0.7) was carried out to highlight the most discriminant compounds. To end, differences in normalized peak areas of *m/z* features were analysed by One-way ANOVA followed by Benjamini-Hochberg multiple testing correction analysis (Benjamini and Hochberg, 1995).

3. Results and Discussion

3.1. Fish condition

In the previous study of Benedito-Palos et al. (2016), data on key performance indicators and gene expression of growth-related markers in liver and skeletal muscle highly supported the suitability of FM/FO replacement by plant ingredients. In agreement with this, when fish coming from this initial trial were randomly sampled for serum metabolomics fingerprinting, all fish showed a similar average body weight ranging between 577 and 612 g (Table 2)**.** Likewise, hepatosomatic index (HSI) and mesenteric fat index (MSI) remained mostly within the normal range of variation for the class of fish size and season (Cruz-García et al., 2009; Benedito-Palos et al., 2010). This revealed a lack of impact of dietary treatment upon body fat storage or tissue lipid trafficking, which are now recognized as clear signs of essential FA deficiencies in gilthead sea bream (Pérez-Sánchez et. al., 2013a; Ballester-Lozano et al., 2015). Despite this, integrative omics approaches combining transcriptomics, proteomics and microbiome analyses highlighted a pro-inflammatory phenotype, with changes in the integrity of the epithelial intestinal barrier and diseases outcomes when fish fed plant-based diets are challenged with bacteria and enteric parasites (Estensoro et al., 2016; Piazzon et al., 2016; 2017). Recently, it has also been proven that plasma levels of sex steroids and the male-female sex reversal through the life cycle of gilthead sea bream are differentially regulated in fish fed marine and vegetable diets (Simo-Mirabet et al., 2018), Nevertheless, sex steroids (testosterone, 11-ketotestosterone, 17ß-estradiol) cannot be considered a major discriminating factor in this study, since their plasmatic concentrations increase gradually through gametogenesis in concomitance with gonadal growth, decreasing abruptly thereafter. Accordingly, circulating sex steroids were almost undetectable in our experimental setup using fish sampled out of the reproductive period, which normally extents for gilthead sea bream from October to March in our latitude (Chaoui et al., 2006; Hadj-Taieb et al., 2013). In any case, our methodology allowed a wide-screening approach, and a total of 12,982 *m/z* features (ions) were obtained in all four acquisition modes (RP and HILIC in both ionization modes ESI+ and ESI-). These numbers are comparable to those previously reported for fed and fasted juveniles of gilthead sea bream, using the same UHPLC-HRMS platform (Gil-Solsona et al., 2017). Of course, not all features corresponded to a single compound, but the number of detectable ions (13,000-15,000) was high enough to have

a wide-representation of the serum fish metabolome. Indeed, the number of different compounds in animal biofluids is estimated to be more than 8,000 (Kałużna-Czaplińska et al., 2014), with around 4,500 in human blood according to the Human Metabolome DataBase (Wishart et al., 2013).

3.2. Untargeted fingerprinting: multivariate analysis

One-way ANOVA was suitable to detect a wide-range of changes in circulating metabolites with more than 5,000 differentially expressed ions when comparing control and extreme D3/D4 groups ($P < 0.05$), but these numbers were drastically reduced after filtering with Benjamini-Hochberg for false positive corrections (Fig. 1A). Thus, the number of ions with a different abundance ranged between 451 and 2,929 when comparisons were made between D1 and D2 fish; and D1 and D4 fish, respectively. However, only four individual features were different between groups D3 and D4, which was indicative that the source of variation when FM/FO diets were supplemented with butyrate was very low in comparison to that of the replacement of FM and FO with plant ingredients alone. This was also evidenced by multivariate PLS-DA analysis as many individuals of D3 and D4 groups overlapped in the score plot (Fig. 1B). This is the reason why data from fish fed D3 and D4 were pooled in the same group (D3/4) for subsequent PLS-DA analyses, where the 71% of variance and 44% of variance was explained or predicted, respectively, by the two first components. The maximum individual variability was achieved within D2 group, but importantly all fish of D1 and D3/D4 groups were correctly classified in the discriminant model. Thus, the maximum separation along both components was found for D1 and D3/4 fish that were distributed along the first (X-axis) and second (Y-axis) component, whereas the separation of D2 and D3/4 fish was only evidenced along the first component reflecting the changes in FO inclusion levels (6.5% D2 diet; 2.50% D3/4 diets). In contrast, the distribution along the second component would primarily reflect the reduced feed intake of FM and fish hydrolysates with inclusion levels of 25.0% in D1 diet and 5.0% in D2, D3 and D4 diets. However, it is noteworthy that the number of features with a $P[corr] > 0.95$ by Orthogonal PLS-DA was reduced to 39, whereas up to 850 ions were identified as clearly discriminant ions in 10-days fasted fish (Gil-Solsona et al., 2017). Therefore, the magnitude of changes induced in the present study by dietary treatment were markedly reduced in comparison to the fasting mediated effects, which suggests that most of them

primarily mirror differences in diet composition rather than functional metabolic dysfunctions associated to changes in diet composition. For this reason, a less restrictive P[corr] > 0.7 was used for the subsequent elucidation procedures.

3.3. Elucidation of untargeted differential compounds

A total of 55 representative compounds with statistically significant changes in abundance after correction for false positives and a VIP score > 1.3 were elucidated (Table 3). Most of them were compounds of lipid nature, such as phosphocholines (PC, 24), lysophosphocholines (LysoPC, 10), free FAs (8) and sphingolipids (2). Other compounds with a different abundance were elucidated as N-acyl-taurines (2), cytidine and cytosine nucleosides (4), cysteinolic acid, tauropine, trimethylamine N-oxide (TMAO), arsenobetaine and hercynine. Accordingly, most of these compounds are related to lipid metabolism and highly reflected the decreased unsaturation index of FAs of vegetable oils. Indeed, FO has an elevated content of n-3 LC-PUFAs, whereas vegetable oils are almost devoid of eicosapentaenoic acid (20:5n-3) and docosahexaenoic acid (22:6n-3), which cannot be synthetized at a high rate in marine fish from the C18 PUFA precursor, α-linolenic acid (18:3n-3) (Tocher, 2015). In consequence, previous studies in gilthead sea bream clearly indicate that the inclusion of vegetable oils in fish feeds reduced the content of LC-PUFAs and increased that of C18 PUFAs in liver, adipose tissue and muscle fillets, with a selective incorporation of unsaturated FAs in polar lipids (Izquierdo et al., 2005; Benedito-Palos et al., 2010; 2013) to preserve and maintain the function of cell membrane surfaces. Especially for fat fish species, most of these changes in the flesh FA composition are highly predictable by means of a dummy regression model (Ballester-Lozano et al., 2014; 2016). Less is known about the effects of diet composition on the FA composition of circulating lipids, although clinical studies evidence that they also reflect the changes in diet composition (Laidlaw and Holub, 2003; Lemaitre et al., 2003) as it was herein the case of circulating PCs, lysoPCs and free FAs. Moreover, the number and degree of these changes in comparison to control group D1 increase with the level of replacement in a dose-dependent manner.

Sphingolipids, as well as phospholipids, are essential components of all eukaryotic cell membranes with important roles in a variety of biological processes including cell division and cell-to-cell interactions (Hannun and Obeid, 2018). In their

simplest forms, sphingosine, phytosphingosine, and dihydrosphingosine serve as the backbones upon which further complexity is achieved. For example, phosphorylation of the C1 hydroxyl group yields the final breakdown products and/or the important signalling molecules sphingosine-1-phosphate, phytosphingosine-1-phosphate and dihydrosphingosine-1-phosphate, respectively (Gault and Obeid, 2010). In the present study, two sphingosine-related compounds were altered by dietary treatment and the abundance of (9-methyl-d19:3) sphingosine was markedly reduced (D2, 23% control fish; D3, 16% control) by the replacement of marine resources by plant ingredients. Conversely, (d14:2) sphingosine was markedly increased in D2 fish (878% with respect to D1 group) with intermediate values with the extreme diet formulation in D3/4 fish, which suggests that other factors that the simple inclusion level of plant ingredients have effects on sphingolipid metabolism, but we are still far to understand the physiological significance of this finding.

In recent years, a number of studies have demonstrated the essentiality of dietary taurine for many commercially relevant species, especially marine teleosts. Consequently, the removal of taurine-rich dietary ingredients such as FM can induce deficiencies with a wide range of symptoms, including reduced growth and survival, increased susceptibility to diseases and impaired larval developments as reviewed by Salze and Davis (2015). However, the paradigm that taurine is an essential nutrient is not nearly as clear in freshwater species and it is difficult to draw definitive conclusions, although the list of fish species for which taurine is required is increasing. In any case, taurine is well recognized as an essential nutrient in most carnivorous fish, and early studies in gilthead sea bream indicated that low levels of taurine in the pool of muscle free amino acids is associated with growth impairments in fish fed plant protein-based diets (Gómez-Requeni et al., 2004). The amides of long-chain FAs with taurine (N-acyl-taurines) are produced via oxidation of bile acid precursors in peroxisomes, and can function as cell signalling molecules with a wide range of biological activities (Hunt et al., 2012). N-acyl-taurines have been recently identified in liver and other rodent tissues, and genetic deletion or pharmacological blockage of the serine amidase FA amide hydrolase (FAAH) causes profound acceleration on wound healing in mouse skin, and repair associated responses in primary cultures of human keratinocytes and fibroblasts (Sasso et al., 2016). In the same study, immunofluorescence images of intact mouse skin show that FAAH co-localizes with cytokeratin 10 and filaggrin, two proteins that are expressed by epidermal supra-basal keratinocytes. In a previous study, we have

identified the cytokeratin 8 as a good marker of multiple aquaculture stressors (tank shaking, sounds, moving objects into water, water reverse flow and light flashes) in the skin mucus of gilthead sea bream (Pérez-Sánchez et al., 2017). The association of cytokeratines with N-acyl taurines has not been established in fish, but we found herein that the concentration of either N-heptadecenoyl-taurine or N-palmitoleoyl-taurine was progressively and consistently reduced with the combined replacement of FM and FO by plant ingredients. This finding opens new research issues in fish nutrition, which would be targeted to alleviate some of the drawback effects of plant-based diets upon the epithelial mucosae of gilthead sea bream, probably mediated by cell renewal or anti-inflammatory processes, as it has been reported for other bioactive compounds, such as butyrate which helps to restore and preserve the integrity and function in gilthead sea bream fed from early life stages with plant-based diets (Estensoro et al., 2016; Piazzon et al., 2017). Moreover, experimental evidence indicates that both butyrate and taurine are able to mitigate through different modes of action the intestinal anomalies of European sea bass fed with highly enriched soybean meal diets (Rimoldi et al., 2016).

Cytidine and nucleoside related compounds (cytosine, deoxycytidine, methylcytosine) were also clear discriminant factors in our experimental model, and their concentrations were consistently increased in fish fed plant-based diets. Intriguingly this was more evident in the group of fish fed D2 diet (200-734% control fish) that in the extreme D3/4 group (120-190% control fish). Since these compounds originate from dietary sources, from cellular excretion subsequent to RNA turnover, from cytosolic pools of nucleotides, or from degradation of nuclear DNA phagocytized by macrophages (Holstege et al., 1984), it is difficult to understand the physiological significance of these findings, although a major source of variation might be related to some kind of cellular DNA instability. Indeed, the highest difference amount control and experimental groups was reported for deoxycytidine and methylcytosine. Degradation of DNA produces deoxycytidine and chemotherapy sharply raises plasma deoxycytidine levels above pretreatment levels (Cohen et al., 1997). At the same time, methylation of cytosines is an important element of epigenetic regulation, and the increased circulating levels of methylcytosine can indicate not only a higher DNA degradation or instability, but also a hyper-methylation at the whole DNA or at specific gene sites. However, this notion needs to be confirmed by more specific assays, because vegetarian life styles are associated with hypo-methylation states (Geisel et al., 2005).

Unlike endogenous compounds, the origin and significance of exogenous compounds with a different abundance was easier to trace, being highly informative of the nature and origin of feed ingredients. Accordingly, the replacement of FM by plant ingredients was associated to a decrease of circulating cysteinolic acid, tauropine, TMAO or arsenobetaine. Cysteinolic acid is a non-protein amino acid similar to taurine, detected in gilthead sea bream and red sea bream (*Pagrus major*) as cholesterol-conjugate precursors in the synthesis of bile salts (Goto et al., 1996; Une et al., 1991). This amino acid is not synthesized by fish, but it can be easily incorporated in the food chain as some marine seaweed such as *Ulva* or *Enteromorpha* contain large amounts (Ito, 1963). Likewise, tauropine is an anaerobic end product found in several marine invertebrate phyla, but widely prevalent in marine molluscs (Venter et al., 2016). The same for TMAO, a compound found in animals, plants and fungi, but the concentration of TMAO in marine animals significantly exceeds that of other organisms (Yancey, 2005). Likewise, arsenobetaine is the arsenic analogue of the quaternary ammonium compound glycine betaine, and marine animals contain very high levels of this compound, non-toxic for human or animals (Molin et al., 2015; Stiboller et al., 2015). Its relative contribution of trophic transfer and biotransformation of arsenic derivatives in the arsenobetaine content in fish is still under debate (Caumette et al., 2012; Popowich et al., 2016), although from our results it was evident the direct relation between dietary FM and circulating arsenobetaine levels.

Another exogenous compound with a high discriminant value in our experimental model was hercynine. This is an intermediate compound in the synthesis of ergothioneine, a natural antioxidant that is only synthesized by non-yeast fungi, cyanobacteria and actinobacteria (Fahey, 2001; Pfeiffer et al., 2011). Therefore, its detectable presence in the serum of fish is indicative of feeding plant ingredients, although its circulating concentration did not parallel the replacement level, being the circulating concentration (arbitrary units) in D2 fish (737 ± 74%) too much higher than that of D3/4 (182 ± 15%) fish. However, when these values were plotted against the relative concentration of hercynine in the diet, a close linear association was found for this compound (Fig. 2). Therefore, with the advent of new formulations, hercynine is coming as good biomarker of raw material traceability, but also of proper feed storage and processing of plant-based diets with no fungi/mycobacteria growth.

448 *3.4. Targeted vitamin analysis*

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450 Vitamins are essential micronutrients that are normally found as precursors of
451 various enzyme reactions in all living cells. However, most of them cannot be
452 synthesized by animals and they need to be obtained exogenously by means of diet
453 fortification, although the use of vitamin-producing microorganisms represents a more
454 natural and consumer-friendly alternative (Le Blanc et. al., 2013). In humans, it has
455 been shown that members of the gut microbiota are able to synthesize vitamin K as well
456 as most of the water-soluble B vitamins, such as biotin, cobalamin, folates, nicotinic
457 acid, pantothenic acid, pyridoxine, riboflavin and thiamine (Hill, 1997). Unlike dietary
458 vitamins, the predominant uptake of the microbially-produced vitamins occurs in the
459 colon (Said and Mohammed, 2016). A similar specialization seems to exist along the
460 digestive tract of fish, as evidenced the microarray gene expression profiling of several
461 genes related to vitamin B_{12} through the intestine of European sea bass (Calduch-Giner
462 et al., 2016). Experimental evidence also indicates that replacement of FM by plant
463 ingredients drives many changes in the micronutrient diet composition, with an
464 important decrease in the content of some vitamins (NRC, 2011). In our experimental
465 model, most of the theoretically mineral and vitamin requirements are met in excess by
466 the diet (Table 1), but to assess the proper levels of circulating vitamins and vitamin-
467 related compounds, a retrospective (targeted) analysis was conducted by means of the
468 MS^E acquisition mode. This approach served to check deficiencies in specific
469 compounds that could have been masked by the astringent Benjamini-Hochberg
470 multiple testing correction in the untargeted approach. Hence, as shown in Table 4, the
471 relative concentration of riboflavin (vitamin B_2) and pantothenic acid (vitamin B_5) were
472 progressively and significantly increased with the replacement of marine sources by
473 plant ingredients in D3/4 fish. Conversely, methylmalonic acid (MMA), used as a
474 biomarker of vitamin B_{12} deficiency in humans and rodents (Watanabe et al., 1991;
475 Carmel, 2011), increased progressively and significantly with the replacement FM/FO
476 by plant ingredients in fish fed D2 and D3/4 diets. The replacement of FM by plant
477 proteins also decreased the concentration of vitamin B_{12} in muscle and liver tissues of
478 Atlantic cod (Hansen et al., 2007), being now well recognized the risk of vitamin B_{12}
479 deficiency in vegetarian humans (Stabler and Allen, 2004; Allen, 2009). Our targeted
480 approach did not detect additional changes in vitamin condition, although vitamin B_7 is
481 markedly reduced by short-term fasting in gilthead sea bream (Gil-Solsona et al., 2017).

All this reinforces the importance to define the core microbiota for a given feeding regime and nutritional status, but studies in livestock animal and fish in particular are still in an infancy state to fully understand the complexity of host and gut microbiota interactions.

4. Conclusions

UHPLC-HRMS approach allowed us to identify a high number of *m/z* ions in the serum of farmed gilthead sea bream. This was the result of combined targeted and untargeted approaches, which identified a wide-range of endogenous and exogenous compounds with a high discriminant capacity as summarized in Fig. 3**.** Multivariate analyses highlighted a clear separation of fish fed the control and plant-based diets, and the distribution through X-axis and Y-axis evidenced the different effects related to FM or FO replacement by plant proteins and oils. Most of the changes reflected the different FA composition of dietary oils in fish growing at high rates without apparent signs of FA deficiencies. However, N-acyl taurines emerged as target compounds to alleviate some of the negative health effects of plant-based diets. Other metabolite changes (cytidine and nucleoside compounds) highlighted different nutritionally-mediated effects on DNA stability and perhaps methylation levels. Targeted vitamin analysis corroborated the risk of low levels of vitamin B_{12} in fish fed plant-based diets, whereas other dietary or microbially-produced vitamins were not affected or increased (B_2, B_5). Lastly, the detection of different exogenous compounds served to trace the use of different raw materials in fish feeds, but also to eventually assess their proper processing and storage.

Disclosures

No conflicts of interest, financial or otherwise, are declared by the authors.

Author contributions

J.V.S, F.H. and J.P.S conceived and designed the experiments. R.G.S, J.C.G., J.N.M., L.L.B. and J.P.S. performed the experiments. All authors have contributed to analysis of data and the final writing of the paper. All authors have read and approved the final manuscript.

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Figure captions

Fig. 1. PLS-DA score plot of acquired data of D1 group individuals (black), D2 (red) and D3/4 (green for D3, blue for D4). Insert is a screen plot of the principal component analysis, showing eigenvalues (blue bars) and cumulative variability explained (orange points) against the number of the principal component.

Fig. 2. Correlation plot of hercynine integrated area in feeds (X-axis) and individual serum samples (Y-axis).

Fig. 3. Integrative profile of differential compounds between D2 and D3/4 compared to control D1 group. Bars show for each dietary group and biological process the number of significantly different ($P<0.5$, ANOVA followed by Benjamini-Hochberg multiple testing correction) abundant compounds. Colors in each bar indicate the level of change (as % of D1) as indicated in the inbox.

Table 1. Ingredients and chemical composition of experimental diets.

Ingredient (%)	Diet			
	D1	D2	D3	D4
Fish meal	23.0	3.0	3.0	3.0
Fish hydrolysate (CPSP)	2.0	2.0	2.0	2.0
Soya protein	16.0	25.0	25.0	25.0
Corn gluten	15.0	25.0	25.0	25.0
Wheat gluten	4.0	7.3	7.3	7.3
Rapeseed cake	12.0	9.7	9.9	9.9
Wheat	11.08	6.80	6.64	6.24
Fish oil	15.60	6.56	2.50	2.50
Rapeseed oil	0.0	4.4	6.5	6.5
Palm oil	0.0	4.4	6.5	6.5
Monocalcium phosphate	0.303	2.097	2.097	2.097
Histidine	0.136	0.136	0.136	0.136
Mineral Vitamin mix [a]	0.5	0.5	0.5	0.5
Cholesterol	0.113	0.113	0.113	0.113
Amino-acid and micronutrient mix [b]	0.20	2.92	2.74	2.74
Antioxidants	0.045	0.045	0.045	0.045
Yttrium	0.03	0.03	0.03	0.03
Butyrate (BP-70)	0.0	0.0	0.0	0.4
Proximate composition				
Dry matter (DM, %)	91.65	91.79	91.80	92.34
Crude protein (%DM)	45.48	46.73	46.12	46.03
Crude fat (% DM)	19.80	19.56	20.13	19.40
EPA + DHA (% DM)	2.90	1.38	0.67	0.63

[a] Supplied the following (g/kg mix, except as noted): calcium 689, sodium 108, iron 3, manganese 1, zinc 1, cobalt 2 mg, iodine 2 mg, selenium 20 mg, molybdenum 32 mg, retinyl acetate 1, DL-cholecalciferol 2.6, DL-α tocopheryl acetate 28, menadione sodium bisulphite 2, ascorbic acid 16, thiamin 0.6, riboflavin 1.7, pyridoxine 1.2, vitamin B_{12} 50 mg, nicotinic acid 5, pantothenic acid 3.6, folic acid 0.6, and biotin 50 mg.

[b] Contains methionine, lysine, choline, and lecithin.

857 **Table 2.** Biometry of sampled gilthead sea bream fed experimental diets. Values are the
858 mean ± SEM (n= 10).

	D1	D2	D3	D4	P-value (ANOVA)
Body weight (g)	611.95 ± 24.2	587.40± 25.8	580.8± 10.7	577.6 ± 21.0	0.679
Liver weight (g)	7.33 ± 0.33	7.42± 0.64	8.06 ± 0.38	7.38 ± 0.38	0.855
Mesenteric fat (g)	13.80 ± 2.18	11.89 ± 2.16	10.61 ± 1.41	10.38 ± 1.50	0.546
HSI (%)[1]	1.20 ± 0.05	1.27 ± 0.06	1.39 ± 0.06	1.28 ± 0.06	0.124
MSI (%)[2]	2.20 ± 0.31	2.19 ± 0.28	1.80 ± 0.20	1.79 ± 0.25	0.673

859 [1]Hepatosomatic index = (100 x liver weight) / fish weight.
860 [2]Mesenteric fat index = (100 x mesenteric fat) / fish weight.

861 **Table 3**. Highlighted compounds obtained from untargeted metabolomics. Values are the mean ± SEM (n= 8-10).

	Compound name	Biological process[†]	Chromatography/ ionization mode	Formula	De/protonated molecule *m/z* (mDa)	RT (min)	D2, % CTRL	D3/4, %CTRL	Corrected P-value[‡]	VIP[††]
1	PC(22:6/16:0)	1	RP(spec) / +	$C_{46}H_{80}NPO_8$	806.5701 (+0.1)	18.86	68 ± 5^b	39 ± 7^c	$1.63E^{-06}$	2.12
2	PC(22:6/18:0)	1	RP(spec) / +	$C_{48}H_{84}NPO_8$	834.6010 (-0.3)	19.86	71 ± 13^b	49 ± 6^c	$3.57E^{-06}$	1.48
3	PC(22:6/18:3)	1	RP(spec) / +	$C_{48}H_{78}NPO_8$	828.5544 (+0.1)	17.65	94 ± 15^a	49 ± 4^b	$1.54E^{-03}$	1.47
4	PC(22:6/20:4)	1	RP(spec) / +	$C_{50}H_{80}NPO_8$	854.5700 (+0.1)	19.05	38 ± 3^b	16 ± 2^c	$7.16E^{-10}$	1.90
5	PC(22:6/20:5)	1	RP(spec) / +	$C_{50}H_{78}NPO_8$	852.5541 (-0.2)	17.54	58 ± 4^b	15 ± 2^c	$1.91E^{-09}$	1.33
6	PC(20:5/14:0)	1	RP(spec) / +	$C_{42}H_{72}NPO_8$	750.5079 (+0.5)	23.44	32 ± 4^b	13 ± 1^c	$1.62E^{-15}$	2.03
7	PC(20:5/16:0)	1	RP(spec) / +	$C_{44}H_{78}NPO_8$	780.5532 (-1.1)	18.45	82 ± 8^b	30 ± 5^c	$4.31E^{-11}$	1.93
8	PC(20:5/16:1)	1	RP(spec) / +	$C_{44}H_{76}NPO_8$	778.5385 (-0.2)	17.88	33 ± 3^b	15 ± 1^c	$1.82E^{-15}$	1.86
9	PC(20:5/18:0)	1	RP(spec) / +	$C_{46}H_{82}NPO_8$	808.5855 (-0.1)	19.5	64 ± 12^b	39 ± 1^c	$8.38E^{-07}$	1.96
10	PC(20:5/18:1)	1	RP(spec) / +	$C_{46}H_{80}NPO_8$	806.5700 (0.0)	18.56	107 ± 14^a	80 ± 6^b	$6.59E^{-02}$	1.47
11	PC(20:5/18:2)	1	RP(spec) / +	$C_{46}H_{78}NPO_8$	804.5541(-0.2)	17.88	51 ± 8^b	20 ± 2^c	$4.98E^{-07}$	1.72
12	PC(20:5/18:3)	1	RP(spec) / +	$C_{46}H_{76}NPO_8$	802.5277 (-1.0)	18.43	125 ± 18^b	63 ± 12^c	$3.84E^{-02}$	1.38
13	PC(20:5/20:4)	1	RP(spec) / +	$C_{48}H_{76}NPO_8$	826.5381 (-0.6)	17.25	92 ± 8^a	46 ± 6^b	$4.98E^{-04}$	1.33
14	PC(20:5/20:5)	1	RP(spec) / +	$C_{48}H_{74}NPO_8$	824.5220 (-1.0)	17.28	43 ± 4^b	9 ± 1^c	$3.26E^{-08}$	1.66
15	PC(18:2/16:0)	1	RP(spec) / +	$C_{42}H_{80}NPO_8$	758.5701 (+0.1)	19.23	410 ± 33^b	571 ± 74^c	$3.07E^{-14}$	2.13
16	PC(18:2/18:0)	1	RP(spec) / +	$C_{44}H_{84}NPO_8$	786.6008 (-0.5)	20.34	625 ± 94^b	1689 ± 270^c	$1.31E^{-05}$	2.12
17	PC(18:2/18:2)	1	RP(spec) / +	$C_{44}H_{80}NPO_8$	782.5711 (+1.1)	18.6	568 ± 68^b	1693 ± 271^c	$3.16E^{-08}$	1.67
18	PC(18:1/16:0)	1	RP(spec) / +	$C_{42}H_{82}NPO_8$	760.5859 (+0.3)	20.03	151 ± 20^b	193 ± 14^c	$2.44E^{-03}$	1.31
19	PC(18:1/18:0)	1	RP(spec) / +	$C_{44}H_{86}NPO_8$	788.6191 (+2.2)	21.22	204 ± 33^b	316 ± 35^c	$6.10E^{-03}$	1.89

20	PC(18:1/18:1)	1	RP(spec) / +	$C_{44}H_{84}NPO_8$	786.6012 (-0.1)	20.31	483 ± 68[b]	761 ± 68[c]	1.10E^{-06}	2.12
21	PC(18:1/18:2)	1	RP(spec) / +	$C_{44}H_{82}NPO_8$	784.5858 (+0.2)	19.37	766 ± 130[b]	1581 ± 285[c]	6.29E^{-09}	2.12
22	PC(18:1/18:3)	1	RP(spec) / +	$C_{44}H_{80}NPO_8$	782.5712 (+1.2)	18.6	488 ± 93[b]	1419 ± 199[c]	6.43E^{-08}	1.67
23	PC(16:0/18:0)	1	RP(spec) / +	$C_{42}H_{84}NPO_8$	762.6013 (0.0)	20.03	517 ± 36[b]	1080 ± 119[c]	1.74E^{-18}	2.16
24	PC(16:0/18:3)	1	RP(spec) / +	$C_{42}H_{78}NPO_8$	756.5545 (+0.2)	18.5	532 ± 85[b]	1317 ± 105[c]	3.84E^{-08}	1.96
25	LysoPC(22:6)	1	RP(spec) / +	$C_{30}H_{50}NPO_7$	568.3405 (+0.2)	9.81	77 ± 15[b]	60 ± 8[b]	1.21E^{-05}	1.55
26	LysoPC(22:5)	1	RP(spec) / +	$C_{30}H_{52}NPO_7$	570.3566 (+0.6)	9.11	143 ± 20[b]	157 ± 25[b]	7.17E^{-03}	1.37
27	LysoPC(20:5)	1	RP(spec) / +	$C_{28}H_{48}NPO_7$	542.3242 (+0.5)	8.58	88 ± 9[b]	87 ± 7[b]	4.21E^{-02}	1.59
28	LysoPC(20:4)	1	RP(spec) / +	$C_{28}H_{50}NPO_7$	544.3386 (-1.7)	8.12	50 ± 10[b]	32 ± 4[b]	2.93E^{-08}	133
29	LysoPC(20:2)	1	RP(spec) / +	$C_{28}H_{54}NPO_7$	548.3714 (-0.2)	7.88	294 ± 50[b]	468 ± 47[c]	1.62E^{-15}	1.61
30	LysoPC(18:3)	1	RP(spec) / +	$C_{26}H_{48}NPO_7$	518.3248 (+0.1)	10.82	212 ± 17[b]	328 ± 66[c]	1.69E^{-14}	1.54
31	LysoPC(18:2)	1	RP(spec) / +	$C_{26}H_{50}NPO_7$	520.3403 (0.0)	9.00	237 ± 28[b]	398 ± 40[c]	5.03E^{-11}	1.71
32	LysoPC(18:1)	1	RP(spec) / +	$C_{26}H_{52}NPO_7$	522.3557 (-0.3)	8.41	138 ± 18[b]	195 ± 29[c]	8.14E^{-07}	1.73
33	LysoPC(18:0)	1	RP(spec) / +	$C_{26}H_{54}NPO_7$	524.3704 (-1.2)	7.55	113 ± 10[b]	170 ± 19[c]	3.78E^{-04}	1.43
34	LysoPC(16:0)	1	RP(spec) / +	$C_{24}H_{50}NPO_7$	496.3402 (-0.1)	10.82	145 ± 26[b]	323 ± 42[c]	3.33E^{-05}	1.38
35	FFA(22:6)	2	RP / -	$C_{22}H_{32}O_2$	327.2316 (-0.8)	15.18	85 ± 10[a]	67 ± 13[b]	3.25E^{-03}	1.31
36	FFA(20:5)	2	RP / -	$C_{20}H_{30}O_2$	301.2167 (-0.1)	15.17	96 ± 17[a]	77 ± 12[b]	4.00E^{-03}	1.55
37	FFA(20:4)	2	RP / -	$C_{20}H_{32}O_2$	303.2316 (-0.8)	15.86	92 ± 12[a]	78 ± 5[b]	9.00E^{-03}	1.77
38	FFA(18:4)	2	RP / -	$C_{18}H_{28}O_2$	275.2004 (-0.7)	14.98	51 ± 7[b]	27 ± 5[c]	7.51E^{-16}	1.95
39	FFA(18:2)	2	RP / -	$C_{18}H_{32}O_2$	279.2316 (-0.8)	15.91	202 ± 38[b]	295 ± 21[c]	6.03E^{-09}	1.95
40	FFA(18:1)	2	RP / -	$C_{18}H_{34}O_2$	281.2472 (-0.9)	15.66	160 ± 18[b]	308 ± 34[c]	1.32E^{-04}	1.45
41	FFA(16:1)	2	RP / -	$C_{16}H_{30}O_2$	253.2161 (-0.7)	16.43	103 ± 19[a]	177 ± 25[c]	3.00E^{-03}	1.35

42	FFA(16:0)	2	RP / -	$C_{16}H_{32}O_2$	255.2316 (-0.8)	16.43	111 ± 18[a]	136 ± 23[b]	1.05E^{-02}	1.40
43	(9-methyl-d19:3) sphingosine	3	RP / +	$C_{19}H_{37}NO_2$	312.2899 (-0.4)	12.32	23 ± 3[b]	16 ± 2[c]	1.71E^{-11}	2.11
44	(D14:2)sphingosine	3	RP / +	$C_{14}H_{27}NO_2$	242.2118 (-0.2)	9.17	878 ± 123[b]	148 ± 27[c]	1.98E^{-04}	2.06
45	N-Heptadecenoyl taurine	4	RP / -	$C_{19}H_{37}NSO_4$	374.2355 (-1.0)	15.08	52 ± 10[b]	22 ± 2[c]	6.04E^{-14}	1.90
46	N-Palmitoleoyl taurine	4	RP / -	$C_{18}H_{35}NSO_4$	360.2209 (0.0)	14.58	47 ± 4[b]	22 ± 3[c]	2.87E^{-12}	1.95
47	Cytidine	5	HI / +	$C_9H_{13}N_3O_5$	244.0941 (+0.8)	4.37	235 ± 28[b]	130 ± 21[c]	1.34E^{-02}	1.39
48	Cytosine	5	HI / +	$C_4H_5N_3O$	112.0502 (-0.9)	4.35	200 ± 26[b]	120 ± 8[c]	1.07E^{-02}	1.78
49	Deoxycytidine	5	HI / +	$C_9H_{13}N_3O_4$	228.0951 (-3.3)	3.48	653 ± 59[b]	190 ± 27[c]	5.62E^{-03}	2.27
50	Methylcytosine	5	HI / +	$C_5H_7N_3O$	126.0645 (-2.2)	4.29	734 ± 103[b]	120 ± 24[c]	3.87E^{-05}	2.13
51	Cysteinolic acid	6,10	HI / -	$C_3H_9NSO_4$	154.0169 (-0.5)	4.05	18 ± 3[b]	10 ± 2[b]	6.51E^{-15}	2.33
52	Tauropine	7,10	HI / -	$C_5H_{11}NSO_5$	196.0286 (+0.6)	2.28	28 ± 4[b]	35 ± 7[b]	1.74E^{-10}	2.32
53	TMAO	7,10	HI / +	C_3H_9NO	76.0760 (-0.2)	5.87	53 ± 10[b]	49 ± 9[b]	2.69E^{-03}	1.52
54	Arsenobetaine	8,10	HI / +	$C_5H_{11}AsO_2$	179.0040 (-1.3)	5.78	50 ± 6[b]	52 ± 7[b]	5.00E^{-05}	1.97
55	Hercynine	9,10	HI / +	$C_9H_{15}N_3O_2$	198.1235 (-0.8)	5.75	737 ± 74[b]	182 ± 15[c]	3.00E^{-06}	2.55

1, Phospholipid metabolism; 2, Fatty acid metabolism; 3, Sphingolipid metabolism; 4, N-acyl amino acid metabolism; 5, Pyrimidine metabolism; 6, Bile acid metabolism/algae amino acid; 7, Anaerobic microbial metabolism; 8, Arsenic metabolism; 9, Fungi metabolism; 10, Exogenous compounds.

‡ANOVA followed by Benjamini-Hochberg multiple testing correction. ††Variable importance in projection measurements in PLS-DA.

865 **Table 4**. Vitamin and vitamin-related compounds obtained from refined targeted approach. Values are the mean ± SEM (n= 8-10).
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Vitamin/vitamin-related compounds		Chormatography/ ionization mode	Formula	De/protonated molecule *m/z*(error mDa)	RT (min)	(%) CTRL D2[†]	(%) CTRL D3/4[†]	P-value (ANOVA)
A	Retinol phosphate	RP/+	$C_{20}H_{31}O_4P$	367.2015 (-2.3)	15.75	140 ± 60[a]	121 ± 63[a]	4.45E-01
B_1	Thiamin	HI/+	$C_{12}H_{16}N_4OS$	265.1118 (-0.5)	5.68	120 ± 18[a]	78 ± 23[a]	2.29E-01
B_2	**Riboflavin**	RP/-	$C_{17}H_{20}N_4O_6$	375.1299 (-0.6)	4.44	144 ± 67[a]	364 ± 132[b]	1.56E-03
B_5	**Pantothenic acid**	RP/+	$C_9H_{17}NO_5$	220.1183 (-0.2)	2.04	120 ± 17[a]	146 ± 25[b]	1.98E-02
B_6	Pyridoxine	RP/+	$C_8H_{11}NO_3$	170.0829 (+1.2)	1.72	96 ± 24[a]	104 ± 21[a]	5.96E-01
B_7	Biotin	RP/+	$C_{10}H_{16}N_2O_3S$	245.0955 (-0.5)	5.36	107 ± 19[a]	120 ± 21[a]	3.68E-01
B_{12}	**Mehtylmalonic acid (MMA)**	RP/-	$C_4H_6O_4$	117.0190 (+0.2)	1.22	195 ± 45[b]	276 ± 35[c]	3.27E-03
C	Dehydroascorbic acid	HI/-	$C_6H_6O_6$	173.0085 (-0.1)	1.12	95 ± 17[a]	122 ± 17[a]	1.03E-01
D_3	25-hydroxyvitamin D_3	RP/+	$C_{27}H_{44}O_2$	401.3412 (-0.8)	13.65	102 ± 39[a]	93 ± 26[a]	3.59E-01
E	α-Carboxyethylhydroxychroman	RP/-	$C_{16}H_{22}O_4$	277.1441 (+0.1)	14.10	106 ± 17[a]	109 ± 15[a]	5.64E-01
K_2	Menaquinone	RP/+	$C_{41}H_{56}O_2$	581.4360 (+0.1)	16.80	72 ± 45[a]	140 ± 54[a]	1.07E-01

867 [†] Percentage of integrated area for the selected compound as a percentage in fish fed control diet (D1). Compounds with statistical significant differences (P< 0.05) against
868 control fish are in bold.
869